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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Patent Application of: Winston T. K. Cheng et al.			
Conf No:	8832	Group Art Unit:	1632
Appln. No.:	10/820,777	Examiner:	Michael C. Wilson
Filing Date:	April 9, 2004	Attorney Docket No.:	683884-2US (CCM0002US)

Title: METHOD FOR PRODUCING BIOLOGICALLY ACTIVE HUMAN FACTOR VIII IN THE MILK OF TRANSGENIC ANIMALS DRIVEN BY MAMMARY-SPECIFIC EXPRESSION CASSETTES

DECLARATION OF CHUAN-MU CHEN UNDER 37 C.F.R. § 1.132

I, Chuan-Mu Chen, hereby declare as follows,

1. I am a joint inventor of the patent application No. 10/820,777 (hereinafter '777 application). I am employed as a Molecular Embryologist and a Professor of the Department of Life Sciences and Institute of Biomedicine at the Chung Hsing University. My current research focuses on the area of gene regulation studies in preimplantation embryo genomes and tissue-specific gene expressions of transgenic animal generations for bio-pharmaceutical productions. I am also interested in research in elucidating the alternative epigenetic modification of DNA methylation change in cancer biology and developmental biology. I have published more than 35 papers and 13 patent applications in embryonic research and cancer research fields (see Annex I provided in my Declaration filed July 17, 2006, in the '777 application). I have been a reviewer for the Taiwan Government National Science Council (NSC) Research Program since 1998, and also an overseas reviewer for the Research Grants Council (RGC) of Hong Kong since 2002. Together with a leading Taiwan transgenic cloned animal research team, I have earned an honor of the Taiwan President Agriculture Innovation Award in 2006.

2. In light of my background and my professional experience, I consider myself and expert and am considered by others as an expert in the fields of research and science mentioned

above. As a result, I am qualified to clarify the non-obviousness of the invention claimed in the '777 application. I have reviewed and understand the claims of the '777 application being submitted with this Declaration.

3. The invention disclosed and claimed in the '777 application is superior and unexpected, thus not obvious, over the prior art, for at least the following reasons:

(A) **The inventors of the '777 application were the first to have successfully generated germline-transmitted transgenic mice, goats and pigs harboring the recombinant human B-domain deleted rFVIII (BDDrFVIII; SEQ ID NO:15) in their genomes.** Several original designs of mammary gland-expressing cassette for exogenic human FVIII gene regulation have been invented in this invention. According to the disclosure of the '777 application, other transgenic mammals, including transgenic cows, expressing the recombinant human B-domain deleted FVIII protein in the milk, could also be successfully generated.

(B) **None of the cited prior art references describes the successful making of BDDrFVIII transgenic mammals or even a reasonable expectation of success for such transgenic mammals.** Those skilled in the art knew that it is difficult and unpredictable to successfully make a transgenic animal having desired characteristics simply based on *in vitro* cell expression studies. Indeed, the uncertainty for successfully making a transgenic mammal that secrets high yield of BDD-rFVIII in transgenic mammals is evident from the following statement in the review paper of Soukharev (2002): "Theoretically, the use of BDD-rFVIII might further increase the yield of FVIII secreted into milk, but there is no information whether transgenic animals of this type have been developed (p. 241)".

(C) **The inventors of the '777 application were the first to actually demonstrate that the use of BDD-rFVIII indeed further increased the yield of FVIII secreted into the milk of transgenic mammals.** The concentration of BDD-rFVIII in the milk of the transgenic mammals was about **250-fold (> 50 μg/mL) more concentrated** than that of normal human plasma (0.2 μg/mL). As summarized in Supplement Table 1, the BDD-rFVIII transgenic mammals claimed in the '777 application secreted higher amounts of BDD-rFVIII protein, at Aver. 50 μg/ml, range from 35-81μg/ml, in milk than any of the transgenic mammals disclosed in the prior art

references, including those in Chen, 2002, which secreted full-length FVIII protein, at Aver. 20 μg/ml, range from 7-50.1 μg/ml, in milk.

Supplement Table 1. Comparison of full-length rFVIII and B-domain deleted rFVIII expression levels in transgenic animal systems

Transgenic Animal	Promoter Used	FVIII cDNA Length	rFVIII Conc. (X fold)	Reference
Tg mice & goat, pig	bovine αLA (2.0-kb)	B-domain deleted FVIII (4.5 kb)	aver. 50 μg/ml range:35-81 μg/ml (~ 250X higher)	'777 Appln.
Tg mice	bovine αLA (2.0-kb)	Full-length FVIII cDNA (7.2 kb)	aver. 20 μg/ml range:7-50 μg/ml (~ 100X)	Our paper; Chen, 2002
Tg sheep	ovine βLG (2.2-kb)	Full-length FVIII cDNA (7.2 kb)	up to 6 ng/ml (~1/50X)	Niemann *et al.*,1999
Tg mice	mouse WAP (2.6-kb)	Full-length FVIII cDNA (7.5-kb)	up to 28 ng/ml (~1/7X)	US patent 5,880,327 Lubon 2001
Tg pig & mice	mouse WAP (2.5-kb)	Full-length FVIII cDNA (7.2 kb)	up to 2.7 μg/ml (~13X)	Paleyanda *et al.*, 1997

(D) **The inventors of the '777 application were the first to discover that the use of BDD-rFVIII also increased the clotting activity of the rFVIII secreted into milk**, see Supplement Table 2. The clotting activity of BDD-rFVIII protein in milk of the transgenic mammals reached a level of about 50-fold higher than that of normal human plasma. Unexpectedly, the inventors further discovered, based on the clotting activity of BDD-rFVIII protein detected in milk of the transgenic mammary glands, that about 10-15% biological activity was detected for the BDD-rFVIII protein secreted in milk compared with ELISA protein quantification. This is higher than the biological activity detected for the full-length rFVIII secreted in milk, such as the 5-10% activity described in Chen, 2002 (page 265).

Supplement Table 2. Recombinant BDD-rFVIII concentration and coagulation activity in the milk of transgenic animals

Transgenic lines	Lactations and days (L/D)	Coagulation activity assay (U/ml)[a]	rFVIII concentration (μg/ml)
BxFVIII-9	L1/D7	57.55 ± 0.96	81.23 ± 2.65
Tg mouse	L1/D14	58.01 ± 1.32	80.87 ± 3.32
(n=4)	L1/D21	45.63 ± 1.56	79.93 ± 2.54
BxFVIII-26	L1/D7	41.77 ± 0.45	52.84 ± 1.12
Tg mouse	L1/D14	46.18 ± 1.36	78.58 ± 3.66
(n=6)	L1/D21	44.89 ± 1.32	72.87 ± 2.73
BxFVIII-3431	L1/D5	28.89	35.12
Tg goat	L1/D10	33.11	50.58
(n=1)	L1/D20	36.87	52.79
BxFVIII-201	L1/D3	30.35	47.34
Tg goat	L1/D11	38.03	55.18
(n=1)	L1/D22	37.90	52.92
	L1/D22	37.90	52.92
		Aver. **10-15% activity**	Aver. **50 μg/ml** (35-81μg/ml)

[a] One unit of rFVIII was defined as equivalent to the amount of human FVIII normally present in 1ml of plasma, approximately 200 ng. Results presented are the average of two independent assays.

(E) **The B-domain deleted rFVIII construction used in transgenic mammals of the '777 application is different from those used in cell line expression systems in the prior art,** see Supplement Table 3. For the cell line expression of BDD-rFVIII protein, there was no need to modify the secretion signal. The native 19-aa secretion signal peptide of the human FVIII is suffice to direct the secretion of the BDD-rFVIII protein in the cell line expression systems. However in constructs used for transgenic mammals, the native 19-aa

secretion signal peptide of the human FVIII must be replaced by a milk-specific signal sequence, such as the bovine α-S1 casein signal peptide or the bovine α-LA signal peptide, to allow efficient secretion of the BDD-rFVIII protein into mammary glands. In addition, a new recombinant spliced site (S741-link to-L1643) was used for a more complete B-domain deletion sequence. See Supplement Table 3.

Supplement Table 3. Comparison of B-domain deletion construction in different cell lines and transgenic animal expression systems

Transgenic animal/cells	Construct name	Signal peptide replacement	B-domain deleted region	Reference
Tg mice & goat, pig	BDD-rFVIII	bovine αS1-casein (15-aa)	Ser741-Leu1643	'777 Appln.
CHO cell	LA-VIII	none	760-1639	Pittman et al., 1993
SK-Hep-1 liver cell	rVIIIB928	none	741-1668	Herlitschka et al., 1998
CHO cell	rFVIII/GC -rAHF	none	741-1563	Oh et al., 2001
COS cell	rFVIII≥B	none	759-1639	Becker et al., 2004
CHO cell	ReFacto	none	744-1639	Wyeth/Genetics Ins., 2001

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these

statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the '777 Application or any patent issued thereon.

Date: Dec. 23, 2007

Chuan-Mu Chen